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Epoetin alfa has potential efficacy in central nervous system disorders

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Abstract

Recombinant human erythropoietin (rHuEPO, epoetin alfa) is a 165-amino acid glycoprotein that has been shown to initiate response to hypoxia and is most widely known for its efficacy in the treatment of a variety of anemias. A series of experiments were conducted using rodent models to investigate the ability of systemically administered epoetin alfa to cross the blood-brain barrier (BBB) and affect the outcome of hypoxia and injury in the central nervous system. Results demonstrated that endogenous erythropoietin (EPO) and EPO receptors are expressed around animal brain capillaries and that systemically administered epoetin alfa crossed the BBB. The epoetin alfa group experienced significantly reduced ($P < 0.01$) tissue damage in an ischemic stroke model when the study drug was administered 24 h before inducing stroke compared with control animals. Significant protective effects of epoetin alfa persisted when epoetin alfa was administered up to 6 h poststroke ($P < 0.05$). Similarly, epoetin alfa reduced trauma-related brain injury when administered 24 h prior to and up to 6 h after blunt trauma when compared with the control group. The volume of tissue necrosis was significantly greater in control animals compared with those that received epoetin alfa ($P < 0.05$). In addition, these studies led to the postulate that epoetin alfa may also have an effect on nervous system inflammation. This was confirmed using an experimental auto-immune encephalomyelitis model, where rats were shown to have significantly delayed onset ($P < 0.01$) and reduced severity ($P < 0.05$) of symptoms after treatment with epoetin alfa. Other studies demonstrated that epoetin alfa had an effect on the latency and severity of seizures and significantly increased ($P < 0.0002$) survival versus controls when mice were exposed to the neurotoxin kainate (used to induce seizures). These findings suggest future potential therapeutic uses for epoetin alfa beyond its anemia-related effects.

Keywords: Epoetin alfa; Anemia; Central nervous system; Blood-brain barrier; Neuroprotection; Hypoxia

1. Introduction

Recombinant human erythropoietin (rHuEPO, epoetin alfa) is a 165-amino acid glycoprotein synthesised by recombinant DNA technology. It has the same amino acid sequence and biological effects as endogenous erythropoietin (EPO), the principal hormone responsible for erythropoiesis [1]. Endogenous EPO is produced primarily in the kidneys in response to hypoxia and circulates through the bloodstream to the bone marrow where it binds to its receptors on erythroid progenitor cells, thereby stimulating red blood cell production. Any decrease in kidney production of EPO results in a deficiency of circulating red blood cells and subsequent anemia.

For years it was believed that EPO was produced exclusively in kidneys and fetal liver. However, recent evidence indicates that EPO mRNA is expressed by other tissues including adult liver (especially in times of stress), brain, lung, testes and spleen. This suggests a wider biologic role for EPO than previously recognized [2–5].

In the central nervous system (CNS), EPO is expressed by both neurons and glia and EPO expression increases in response to hypoxia [6,7]. Expression of the EPO receptor (EPO-R) has been detected in embryonic mouse brains at levels comparable to those in adult mouse hematopoietic tissue [8]. However, these levels diminish with development and are not readily detectable at birth [8]. These observations led to further studies,

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demonstrating that both human and mouse EPO-R genes are actively transcribed, not only in hematopoietic tissue, but also in the developing embryonic brain, suggesting that EPO-R may play an important role in the development of select nonhematopoietic tissue [9]. Other studies showed that EPO and EPO-R are localized in specific areas of embryonic, fetal and adult brains of rats, monkeys and humans. Their persistence in the mature brain is consistent with roles for EPO in both CNS development and homeostasis [10–12]. Additionally, EPO and EPO-R mRNA have been detected in biopsy material from the hippocampus, amygdala and temporal cortex from humans as well as from various brain regions of monkeys. In animal studies, experiments with low levels of oxygen have resulted in elevated levels of EPO mRNA [6,13]. Expression of EPO and the EPO-R in the brain (particularly in such oxygen-sensitive areas as the hippocampus and cerebral cortex), regulation of their expression during fetal development, and increased expression of EPO and EPO-R under hypoxic conditions led to the hypothesis that rHuEPO could act as a neurotrophic and neuroprotective factor with possible therapeutic potential in cases of brain damage due to hypoxia, ischemia or brain hemorrhage [14].

2. Neuroprotective function of EPO

Results of *in vitro* and *in vivo* studies have provided evidence of a neuroprotective effect of exogenous EPO. Glutamate is an excitatory amino acid neurotransmitter that mediates neuronal damage produced by hypoxia/ischemia [5]. *In vitro*, EPO had been shown to protect cultured rat hippocampal and cerebral cortical neurons from glutamate toxicity [7,15,16]. It has also been shown that EPO protects neuronal cultures from nitric-oxide-induced death [16], rescues neuroblastoma cells from hypoxia-induced apoptosis [17], and prevents the death of cultured hippocampal neurons from newborn rats when administered at the onset of hypoxia [18].

In vivo, EPO infusion into the cerebroventricles of stroke-prone spontaneously hypertensive rats has been shown to ameliorate ischemia-induced place-navigation disability, cortical infarction, and thalamic degeneration [19]. In a gerbil model of global cerebral ischemia, infusion of EPO into the lateral ventricles prevented ischemia-induced learning disabilities and rescued hippocampal neurons from lethal ischemic damage [16]. Also, intraventricular injection of recombinant mouse EPO resulted in a significant reduction in infarction volume in mice with induced cerebral ischemia [7].

These observations set the stage for investigation of the possible therapeutic effects of EPO in CNS disorders. Direct delivery of EPO into the CNS intrathecally or via the intracerebroventricular system had been shown to be neuroprotective in stroke models, but this route of treatment is clinically impractical. Systemic delivery to achieve CNS effects was not considered at that time, since it had always been assumed that large molecules such as EPO could not cross the blood-brain barrier (BBB). However, in a preclinical study characterising the human BBB transferring receptor, covalent coupling of a neuropharmaceutical with a brain transport vector resulted in transportation across the BBB [20]. Specific vectorial movement of macromolecules across the BBB begins by binding of the macromolecule to receptors on the luminal surface of endothelial cells. This initiates endocytosis and subsequent translocation of the macromolecule across the BBB [21]. In other studies, aluminum enhanced the permeability of the BBB to certain hormones, the greatest effect being seen with thyroxine, which crosses the BBB by carrier-mediated transport [22].

Against this background, a series of studies using rodent models was initiated by Brines and colleagues to investigate the expression of EPO and EPO-R in normal brain tissue and the ability of a commercially available rHuEPO (epoetin alfa) to cross the BBB [23,24]. Subsequently, four additional rodent-model studies were conducted in which epoetin alfa was administered systemically to assess its effect on neuroprotection in focal ischemic stroke, reduction of blunt trauma injury, reduction of the clinical severity of neurologic inflammation and the latency and severity of seizures.

2.1. Erythropoietin and its receptor in the normal brain

The first study investigated the expression pattern of EPO and EPO-R in the brain [23,24]. Human, mouse and rat tissues were stained using polyclonal antibodies for immunohistochemical staining. The most intense immunoreactivity for EPO-R was found in the frontal cortex and hippocampus (Fig. 1). Specifically, EPO-R was expressed to a large extent in many medium and large neurons (wherein it was localized to the somata and proximal dendrites), as well as capillaries, particularly within white matter. High-power microscopic examination revealed that capillaries were enveloped by numerous EPO-R immunopositive processes derived from nearby astrocytes. Transmission electron microscopy confirmed that the predominant EPO-R immunoreactivity was located in astrocyte foot processes surrounding capillaries and was also present within or on the surface of endothelial cells. There was no significant expression of EPO-R in larger vessels and most astroglia.

2.2. Epoetin alfa crossed the BBB

The previous study demonstrated high expression of capillary EPO-R at the BBB, suggesting that epoetin

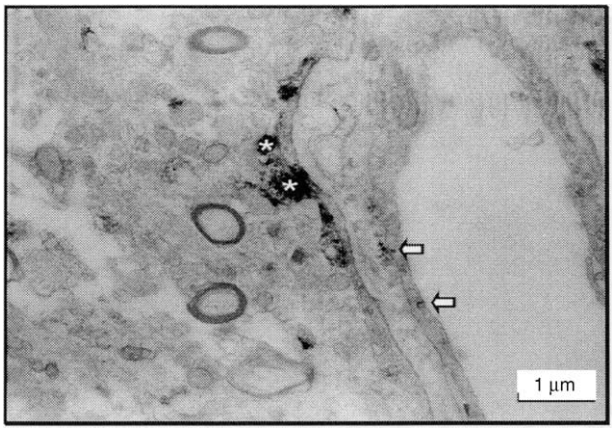

Fig. 1. EPO-R immunoreactivity within the astrocyte foot processes surrounding the capillaries (*asterisks*), and on the surface of endothelial cells (*arrows*) in brain tissue. Reprinted with permission [23,24].

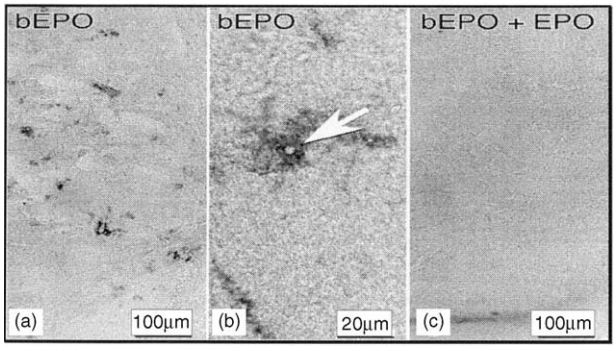

Fig. 2. Observations of biotinylated epoetin alfa activity after intraperitoneal injection into mice in brain sections at 5 h (panels A and B), and after combination with 100 times excess of unlabeled epoetin alfa (panel C). Reprinted with permission [23,24].

alfa administered to the systemic circulation was directly transported into the CNS. To confirm these observations, biotinylated epoetin alfa was injected intraperitoneally (i.p.) into mice, and using the streptavidin-peroxidase methodology, epoetin alfa was visualized in brain sections at 5 and 17 h after injection [23,24]. These time points were chosen based on the pharmacokinetics of a single 5000-IU/kg epoetin dose given i.p., where peak activity occurred at 5 h and serum levels decreased to $<1\%$ of the peak concentration after 17 h.

After 5 h, biotinylated epoetin alfa was detected around capillaries and extended into brain parenchyma. When excess unlabelled epoetin alfa was injected together with the labelled epoetin alfa, there was little or no reactivity around capillaries (consistent with a specific and saturable transport mechanism) (Fig. 2). After 17 h, no labelled epoetin alfa was detected around capillaries but, rather, was scattered around neurons. Separately, significant levels of labelled epoetin alfa were detected in cerebrospinal fluid obtained from cisterna magna of male rats. These findings strongly suggest active translocation of epoetin alfa across the BBB that specifically targets neurons.

2.3. Systemically administered epoetin alfa is neuroprotective against focal ischemic stroke

The next study was designed to determine whether epoetin alfa has protective effects against focal ischemic stroke [23,24]. Stroke was induced in male rats by permanently occluding the middle cerebral and right carotid arteries and then occluding the left carotid artery for 1 h. (This procedure produces a penumbra {area at risk} comprising an initially small necrotic core and a large surrounding volume of potentially viable neurons within the right frontal cortex. Ischemia, if untreated, triggers apoptosis of susceptible neurons within the penumbra over time.) Epoetin alfa, 250–5000 IU/kg, was injected i.p. 24 h before occlusion of the left carotid artery; simultaneously with occlusion; or 3, 6 or 9 h after occlusion. A control group of animals was injected with saline at the same time points.

After 24 h, brain tissue was harvested and living tissue was distinguished from dead tissue using computerized volumetric analysis of triphenyltetrazolium reduction. Rats receiving epoetin alfa 24 h before and up to 3 h after occlusion exhibited significantly less damaged tissue than did controls ($P<0.01$). Although the protective effect diminished with time, after 6 h, significant protection was still detected ($P<0.05$). There was no protection observed when epoetin alfa was given 9 h after occlusion (Fig. 3). The lowest dose that offered protection was 450 IU/kg. This window of protection was attributed to the ability of epoetin alfa to induce protective genes in potentially viable cells within the ischemic penumbra before they underwent apoptosis.

2.4. Epoetin alfa reduced injury by blunt-force trauma

Mechanical insult of the brain can result in ischemic, inflammatory and excitotoxic injury, as well as cavitary lesions. The potential role of epoetin alfa in reducing traumatic injury created by blunt force to the brain was investigated using rodent models [23,24]. A calibrated piston was used to effect a blow to the intact calvaria of the frontal cortex of anesthetized animals. Epoetin alfa was injected i.p. at a dose of 5000 IU/kg. Injections were given at 24 h before the blow or at 0, 3 or 6 h after the blow, and the doses were continued for 4 days. Ten days after the blow, brains were harvested, sectioned and stained for analysis.

There was less cavitary injury in brains from animals who received epoetin alfa 24 h prior to and up to 6 h after assault compared with the control group of animals who did not receive epoetin alfa. Quantitative analysis indicated a greater protective effect in the epoetin alfa group. The volume of necrosis was significantly greater

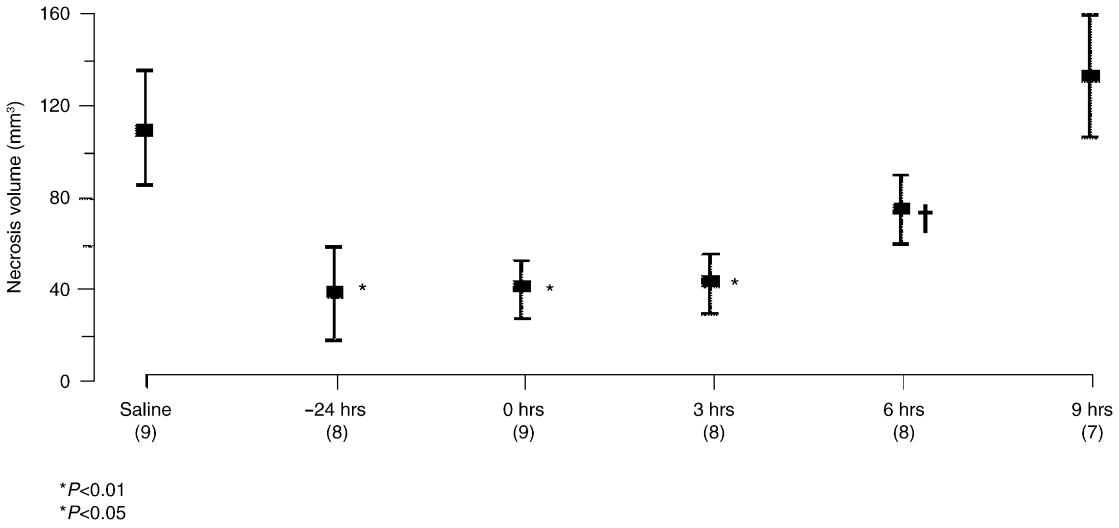

Fig. 3. Effect of systematic administration of epoetin alfa on infarct volume after cerebral occlusion in the rat at 24 h before occlusion, 6 h after occlusion and 9 h after occlusion. Reprinted with permission [23,24].

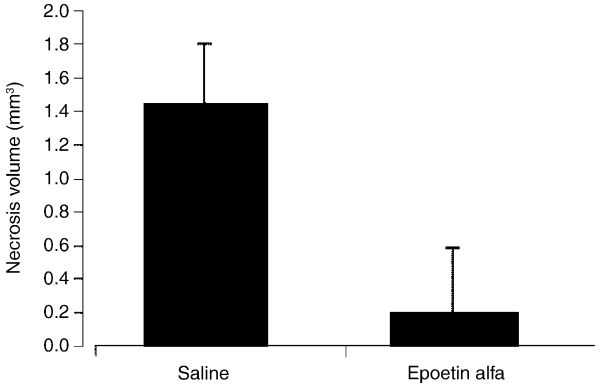

Fig. 4. Protective effect of epoetin alfa administered 24 h before blunt-force injury to the frontal cortex. Reprinted with permission [23,24].

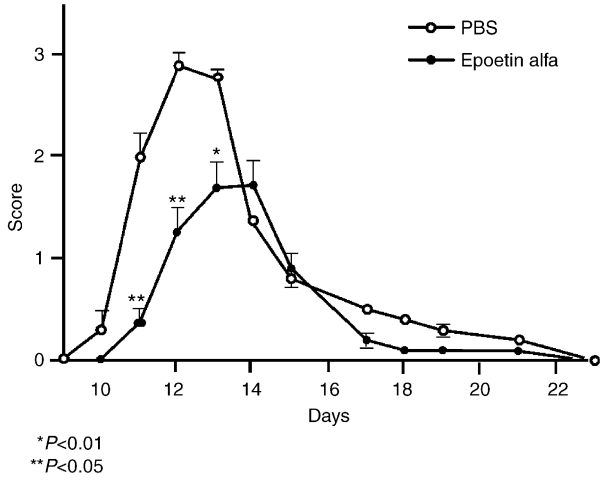

Fig. 5. Epoetin alfa significantly delayed the onset and reduced the severity of experimental autoimmune encephalomyelitis symptoms. Reprinted with permission [23,24].

in the control animals than in the group that received epoetin alfa ($P < 0.05$) (Fig. 4). Additionally, in the control animals, the region immediately surrounding the necrotic core contained a large population of mononuclear inflammatory cells, whereas in the epoetin-alfa-treated animals, this region showed markedly reduced inflammatory infiltrate, suggesting an immunomodulatory role for epoetin alfa.

2.5. *Epoetin alfa reduced the clinical severity of experimental encephalomyelitis*

The observation that epoetin alfa treatment reduces inflammatory infiltrate after trauma led to the postulate that epoetin alfa might reduce nervous system inflammation caused by other pathologic processes.

A rat model of experimental autoimmune encephalomyelitis was created to test this hypothesis [23,24]. Experimental autoimmune encephalomyelitis was induced in female Lewis rats by immunisation with guinea pig myelin basic protein and complete Freund adjuvant. Immunization induced clinical symptoms after 10 days, and severe paralysis was observed after day 12. Three days after immunization, two groups of rats received either epoetin alfa (5000 IU/kg) or saline, administered daily, with injections continuing for 15 subsequent days. Epoetin alfa significantly delayed the onset and reduced the severity of experimental autoimmune encephalomyelitis symptoms compared with controls ($P < 0.01$ and $P < 0.05$, respectively) (Fig. 5). No rebound of symptoms occurred 3 weeks after termination of epoetin alfa therapy, in contrast to the occurrence of rebound that has been noted following discontinuation of treatment in groups receiving other therapies, such as glucocorticoids or interferon-beta [25]. Whether the effect of epoetin alfa in this model was

on the inflammatory system or immune system, or both, is as yet unknown.

2.6. Effects of epoetin alfa on latency and severity of seizures induced by kainate

Excitotoxicity accompanies many different forms of brain injury [26], and it was postulated that epoetin alfa might reduce such toxicity. A mouse model was designed to test this hypothesis by examining the effects of epoetin alfa on toxicity caused by kainate, a glutamate analogue whose toxicity generally results in seizures [23,24]. First, the relationship among systematic doses of kainate, its toxicity and the latency and severity of seizures were determined. It was discovered that when kainate was administered at the median effective dose (ED_{50}) of approximately 20 mg/kg, death caused by status epilepticus occurred some 18 min later.

As in previous experiments conducted in mouse models, epoetin alfa was administered 24 h before the administration of kainate. Mice receiving epoetin alfa had delayed onset of status epilepticus and reduced motor involvement compared with control mice. Mortality was also reduced by approximately 45% (Fig. 6). In the epoetin alfa group, mean survival time increased significantly compared with the control group (25.8 min versus 18.2 min, $P < 0.0002$). At doses below ED_{50} of 20 mg/kg, kainate produces behavioral seizures rather than status epilepticus. The seizure activity induced by this amount of kainate was significantly reduced by administration of epoetin alfa 24 h earlier. However, epoetin alfa administered 30 min before or after kainate exposure offered no protective effect from seizure activity. The seizure prevention of a single dose of epoetin alfa at 5000 IU/kg lasted for at least 3 days, unlike conventional antiepileptic agents, which require continued administration after terminating seizure episodes to be effective (Fig. 6). Epoetin alfa apparently has a mode of action on seizure activity different from that of conventional anti-epileptic drugs.

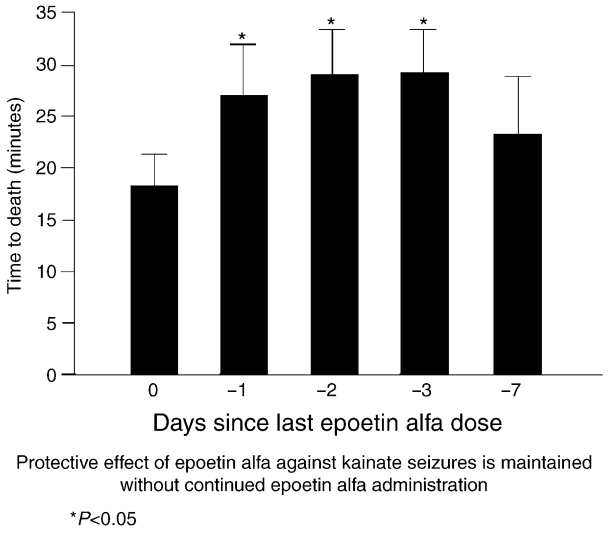

Fig. 6. Epoetin alfa delays and lessens kainate-induced seizure. Reprinted with permission [23,24].

2.7. Effects of epoetin alfa on spinal cord ischemic injury

Erythropoietin and its receptor are expressed by human neuronal and glial cells in the fetal spinal cord [17]. These observations have led to several experimental studies investigating whether epoetin alfa exerts a neuroprotective effect in spinal cord ischemia similar to that exerted in the ischemic brain.

In an *in vitro* study, application of rHuEPO protected fetal rat spinal motoneurons from apoptosis induced by brain-derived neurotrophic factor deprivation or kainic acid exposure [27]. To expand these results and those obtained in the studies of Brines and his colleagues [23,24], the possible neuroprotective and ameliorating effects of epoetin alfa were investigated in an experimental transient global spinal cord ischemia model in rabbits [28]. Spinal cord ischemia was produced by occlusion of the abdominal aorta for 20 min. Saline or epoetin alfa (350, 800 or 1000 IU/kg) was administered intravenously immediately after the onset of reperfusion. Epoetin alfa–treated animals, compared with those given saline, exhibited better neurologic function after 1 h of reperfusion. Neurologic status in all epoetin alfa–treated animals continued to improve over the next 48 h, whereas saline-treated animals showed no improvement (difference, $P < 0.05$). Histologic examination of the spinal cord indicated widespread motor neuron injury and a prominent inflammatory infiltrate in the saline-treated but not in epoetin alfa–treated animals. This finding confirmed the neuroprotective effects of epoetin alfa on spinal motoneurons against ischemic insult. Moreover, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) showed many positive large motor neurons in the ventral gray matter of control animals but only rare reaction product in the gray matter of epoetin alfa–treated animals—a finding that strongly supports an apoptosis-blocking effect of epoetin alfa in this class of neurons. These findings suggest that epoetin alfa has both an acute and a delayed beneficial effect on ischemic spinal cord injury and acts through multiple mechanisms.

3. Mechanisms and clinical implications of EPO neuroprotection

It is well established that normalizing hemoglobin and hematocrit levels with rHuEPO in uremic patients and those with cancer- or treatment-related anemia can maintain or improve quality of life and possibly also cognitive function [29–34]. Additionally, results of *in*

vitro and *in vivo* studies indicate that rHuEPO may also provide neuroprotective benefits independent of its hematologic effects. A number of mechanisms have been proposed for the neuroprotective effects of rHuEPO including repression of apoptosis either by maintenance of Bcl-2 or Bcl-x_L expression, as occurs with erythroid precursor cells [5] or by inactivation of caspases [35,36] via an EPO-induced increase in intracellular calcium. Calcium influx into neuronal cells leads, in turn, to a sustained increase in neuronal nitric acid, which inhibits caspase function [36]. Erythropoietin may also provide neuroprotection by inhibiting glutamate release, increasing glutamate uptake, or desensitizing glutamate receptors. It is also possible that EPO upregulates enzymes that scavenge oxygen radicals [5] or reduces inflammation at the site of injury either directly via inhibition of a member of the inflammatory cascade (e.g. caspase) or indirectly by prevention of apoptosis [27].

The observed neuroprotective effects of epoetin alfa in several different models of neuronal injury suggested target CNS diseases/disorders that might benefit from such therapy. For example, in the model of ischemic stroke [23,24], administration of epoetin alfa prevented apoptosis of neurons within the ischemic penumbra and decreased the infarct volume. In the clinical setting, such an effect in ischemic-stroke patients could translate into less extensive injury and preservation of function (e.g. speech, motor). In two other models of CNS injury, e.g. blunt trauma [23,24] and spinal cord injury [28], epoetin alfa attenuated neuronal injury and also markedly reduced the inflammatory infiltrate. The anti-inflammatory effect is especially noteworthy, as both forms of injury are characterized by intense inflammation, which can have dire clinical consequences. In patients experiencing such injury, much of the neurological dysfunction occurs as a result of disruption of the white matter (ascending and descending long tracts), particularly because of reactive inflammation-driven oligodendrocyte apoptosis [37]. Epoetin alfa therapy also ameliorated the latency and severity of kainate-induced seizures—a model of temporal lobe epilepsy [23,24].

Based on its multiple actions, other target diseases/disorders for which epoetin alfa therapy may afford a potential benefit are amyotrophic lateral sclerosis (ALS), Alzheimer's disease and Parkinson's disease. Some known contributors to neuronal damage in these diseases that could possibly be modulated by epoetin alfa include excitotoxic damage and oxidative stress in ALS, oxidative stress, free radical damage, glutamate-related toxicity in Parkinson's disease and glutamate-mediated toxicity in Alzheimer's disease [38].

Given the encouraging results obtained in animal models, several clinical studies of the neuroprotective effects of rHuEPO are ongoing or under consideration. In a pilot study conducted in Germany (the Göttingen EPO-Stroke Trial), 40 ischemic stroke patients were given intravenous injections of saline (control group, $n=20$) or rHuEPO ($n=20$) within 5 h of onset of symptoms [39]. The cerebrospinal fluid EPO level increased to 60–100 times that of the control group of patients, demonstrating that intravenously administered EPO reaches the brain. The treated patients subsequently had earlier as well as greater improvement in follow-up and outcome scores. They also tended to have smaller areas of damaged brain tissue, as measured by MRI, 1 month after their strokes. In view of these encouraging results, the study has been expanded into a multicenter trial including four centers. In another ongoing trial being conducted at eight centers, the effect of epoetin alfa on neurodegeneration is being assessed in double-blind fashion in schizophrenic patients receiving rHuEPO in addition to their symptomatic neuroleptic medication [40].

4. Conclusions

It has been demonstrated that EPO-R is expressed in other cells besides erythroid progenitors and that epoetin alfa may have significant effects on disease states other than anemia. It was postulated that the drug has a potential for treatment of CNS disorders, and the studies described were designed initially to examine whether peripherally injected epoetin alfa could cross the BBB. These experiments showed that peripherally injected epoetin alfa crossed the BBB and protected brain tissue from a variety of insults. The presence of EPO-R on brain capillaries suggests specific translocation of EPO; however, the underlying biochemical mechanism for neuroprotection requires further investigation.

The results of the rodent-model studies suggest that epoetin alfa may be effective as a therapy in various CNS-specific illnesses such as ischemic stroke, epilepsy, Parkinson's disease, Alzheimer's disease and amyotropic lateral sclerosis. Further study of future clinical uses is warranted.

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